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Experimental evidence of warming-induced flowering in the Mediterranean seagrass *Posidonia oceanica*

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ABSTRACT

Sexual reproduction in predominantly clonal marine plants increases recombination favoring adaptation and enhancing species resilience to environmental change. Recent studies of the seagrass *Posidonia oceanica* suggest that flowering intensity and frequency are correlated with warming events associated with global climate change, but these studies have been observational without direct experimental support. We used controlled experiments to test if warming can effectively trigger flowering in *P. oceanica*. A six-week heat wave was simulated under laboratory mesocosm conditions. Heating negatively impacted leaf growth rates, but by the end of the experiment most of the heated plants flowered, while controls plants did not. Heated and control plants were not genetically distinct and flowering intensity was significantly correlated with allelic richness and heterozygosity. This is an unprecedented finding, showing that the response of seagrasses to warming will be more plastic, more complex and potentially more resilient than previously imagined.

1. Introduction

Climate change is a global experiment in adaptive capacity, as species tolerate, adapt or die with changing conditions. While increasing temperatures are pushing many species to the very limit of their tolerance, it is also revealing a previously unimagined plasticity in the response of others. For most ecosystems dominated by primary producers, how plant species respond to rapid environment change is going to be critical in determining the future of these ecosystems. In this, flowering plants have a large advantage. The ability to switch between vegetative and reproductive modes of growth has been central to the remarkable evolutionary success of flowering plants, allowing them to adapt to changing environmental conditions. Sexual reproduction not only favors genetic variation and, hence, the generation of new genotypes that can potentially colonize new habitats or resist environmental changes, but also the production of seeds that facilitates dispersal and escape in time and space (e.g. Williams, 1975; Venable and Lawlor, 1980).

In terrestrial plants flowering is regulated either autonomously or by environmental factors and is controlled by a variety of interrelated mechanisms (Hempel et al., 2000). In most angiosperms, light and temperature are the principal environmental triggers for switching from vegetative to reproductive growth (Johansson et al., 2013). Most temperate perennial grasses require a dual induction for flowering, a primary induction characterized by low temperatures (vernalization) and short light photoperiods, and a secondary induction characterized by the transition to long days, enhanced by moderately high temperatures (Heide, 1994). In addition to photoperiodic flowering and vernalization, flowering can also be induced as a plant stress response (Takeno, 2016). Diverse stress factors, including high or low light intensity, UV radiation, high or low temperature, drought, nitrogen deficiency, among others, have been reported to induce flowering (Takeno, 2016). Stress-induced flowering has been suggested to be an evolutionary strategy to maximize the survival under adverse conditions (Kazan and Lyons, 2016).

In clonal species, such as seagrasses, the frequency of sexual reproduction varies considerably among and within species and plays an important role in determining the demographic and genetic structure as

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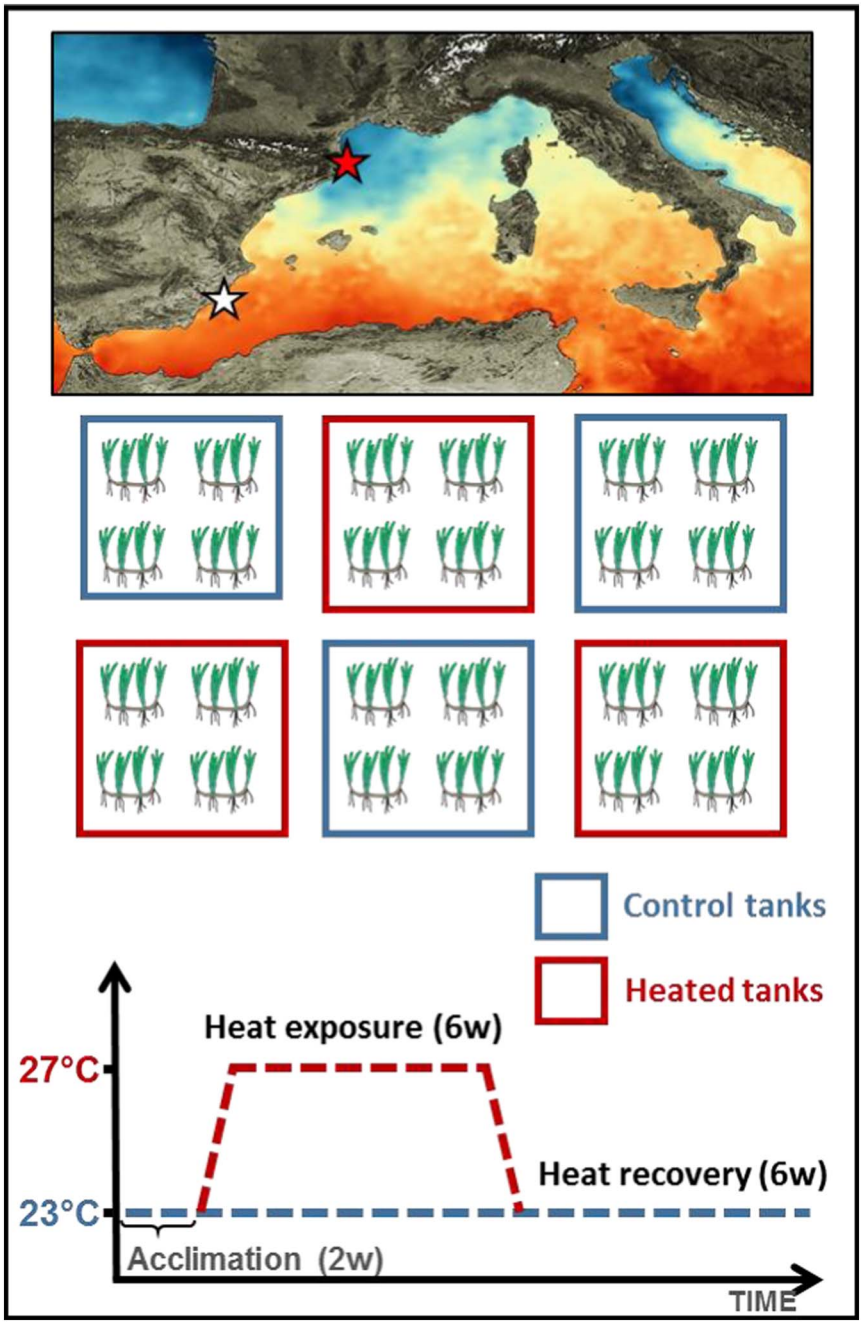

Fig. 1. Map showing the location with a red star of the *Posidonia oceanica* meadow where experimental plant fragments were collected (top panel; modified from the European Space Agency; http://www.esa.int/spaceinimages/Images/2008/10/Mediterranean_sea_surface_temperature). Blue colours represent colder waters and red colours warmer ones. The white star indicates the location of the Oceanography Center of Murcia (Spanish Oceanography Institute) where the mesocosms facilities are located. Experimental setup, showing the six experimental tanks (three heated and three control tanks) each with 4 plant fragments (central panel) and temperature treatment and duration (bottom panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

well as the genetic diversity of populations (Procaccini et al., 2007; Kendrick et al., 2016). Genetic diversity is important to maintain the resilience of seagrass meadows to disturbances (Reusch et al., 2005; Reynolds et al., 2013, 2016). Sexual reproduction also generates dormant stages (e.g. seeds) that are essential for some species to tide over severe disturbances (Jarvis and Moore, 2010; Unsworth et al., 2015). Besides its importance for the maintenance and adaptation of natural populations, the reproductive biology of seagrasses is one the least known aspects of this particular group of plants (Ackerman, 2006). Flowering can occur through the year in some species (e.g. *Halophila ovalis*), while in others it may be highly seasonal, and with variable frequency (McMillan, 1976). The biological basis for these patterns of sexual reproduction as well as the environmental cues inducing flowering in seagrasses are almost unknown (De Cock, 1980; Jahnke et al., 2015; Lee et al., 2005; McDonald et al., 2016). The endemic Mediterranean species *Posidonia oceanica* uses vegetative growth as its principal means of expansion and sexual reproduction is highly variable in space and time (Balestri, 2004), starting in September–October and coinciding with cooling water and shortening day length. Flowering varies greatly across spatial and temporal scales; a long-term study of 54 meadows showed that the percent of flowering meadows ranged from 80% to 3%, from 1980 to 2005 (Diaz-Almela et al., 2006). Within single meadows, only a low percent of shoots flower, and the values vary greatly among regions and meadows (Balestri, 2004; Diaz-Almela et al., 2006, 2007).

Although infrequent and variable, flowering of *P. oceanica* may well be an effective adaptive mechanism for this species to weather the uncertainties of a changing climate. This slow-growing, millenary seagrass dominates the Mediterranean benthos, and is highly sensitive to a range of anthropogenic threats. How *P. oceanica* responds to frequent heat waves that increasingly define the new normality of a changed climate (Schar, 2016) will largely determine the continued fate and

functioning of this valuable ecosystem and of the entire coastal habitat. Based on observed plant mortalities in natural populations from the Balearic Islands and climate forecast models, gloomy perspectives predicted a major decline of this ecosystem in the coming decades (Jordà et al., 2012). However, the plant has a suite of potential adaptation strategies at physiological and molecular levels, to face variable temperature regimes in coastal zones. Indeed, plants from shallow meadow stands and warmer environments have higher physiological tolerance to transient warming than deep plants (Marín-Guirao et al., 2016), thanks to their exclusive ability to activate molecular mechanisms involved in thermal tolerance (Marín-Guirao et al., 2017). Furthermore, long-term observations from the field indicate that flowering in *P. oceanica* meadows increases in unusually warm years, suggesting that temperature could potentially induce flowering events (Diaz-Almela et al., 2007), and serve as a useful adaptive response to changing temperatures. Despite the evidence of these correlational observations, the precise mechanism linking higher temperatures with flowering remains unclear. A range of environmental, demographic or genetic factors have been proposed as controlling flowering, among them shoot age (Balestri and Vallerini, 2003), resource availability and kin relationships (Jahnke et al., 2015) and sunspot numbers (Montefalcone et al., 2013). Moreover, a recent transcriptomic analysis of field-collected *P. oceanica* inflorescences has suggested the importance of environmental factors (i.e. light and temperature) in regulating flowering in the species (Entrambasaguas et al., 2017). Unambiguously determining which of these factors triggers flowering requires carefully experimentation, which is often difficult given the complexities of maintaining these slow-growing species in controlled conditions. We used mesocosm experiments to determine if unusually high temperatures (such as those experienced during heat waves) could predictably induce flowering in *P. oceanica*, and if this flowering has a shoot-specific genetic basis.

2. Materials & methods

We assessed *P. oceanica*'s response to transitory heat stress, simulated under laboratory mesocosm conditions. Divers collected large plant fragments at the beginning of June from a 7 m deep meadow from the NE coast of Spain (Montgó cove, 42° 06′ 23″ N/3° 10′ 16″ E; Fig. 1). This region is close to the coldest sector of the Western Mediterranean basin with summer daily temperatures ranging between 18.3 and 25 °C (90% of the days between 20 and 23 °C). Plants were transplanted in the mesocosm system located at the Oceanography Center of Murcia within 36 h of collection. Apical plant fragments of similar size and with a high number of interconnected vertical shoots (Table 1) were individually planted in plastic pots filled with coarse washed-sediments and randomly placed in six independent 500-l aquaria, each housing four pots as depicted in Fig. 1. Each aquarium was integrated in a circuit of circulating seawater with a 500 l reservoir tank, in which water flow, temperature, light, salinity, pH and nutrients were controlled. Water quality was controlled through continuous physical and chemical filtration, periodic water changes and analyses of nutrient concentrations (mainly nitrogen and phosphorous). Irradiance was provided with a 400 W metal halide lamp (Aqua Medic aqualight-400). Water temperature was controlled with a highly precise (± 0.1 °C) automated system consisting of a water circuit connected to a refrigerator (5 °C) and a heater (40 °C) coupled to temperature sensors placed in each aquarium. We monitored pH continuously with pH electrodes (Aqua Medic AT-Control) and monitored salinity using a WTW conductivity meter (model Cond. 197i). This mesocosm system has been demonstrated to be very effective in culturing *P. oceanica* (e.g. Marín-Guirao et al., 2011, 2012; Sandoval-Gil et al., 2014).

Plants were acclimated for 2 weeks with the summer mean ambient conditions recorded at the sampling site: salinity of 37.5 psu, temperature of 23 °C and irradiance of 300 μmol $m^{-2} s^{-1}$ under a 12 h:12 h light:dark photoperiod. After acclimation, water temperature in three, randomly chosen experimental tanks was increased to 27 °C (+ 4 °C above control mean) for six weeks and subsequently returned to control levels to allow plants to recover from heat stress for another six weeks (Fig. 1). This temperature was chosen to reflect the temperatures plants would likely experience during extreme heat waves in shallow Mediterranean waters. In addition, to avoid possible effects of potential gradients between samples, we randomly permutated plant positions every 2–3 days.

Plant growth was measured both during the exposure as well as the recovery period using the Zieman method (Zieman, 1974). At the start of the experiment, 2–3 shoots per plant fragment were marked by punching a hole above the ligule of the outermost leaf with a hypodermic needle, and tagged with a plastic clamp. Marked shoots were collected at the end of the experimental period to measure newly produced tissue to estimate shoot leaf growth rate (cm^2 $shoot^{-1}$ day^{-1}). Average growth for each plant fragment was tested using a nested ANOVA for the treatment effects, being the 500 l tanks the factor nested within each treatment.

If present, the flowers (inflorescences) were counted and flowering intensity was expressed as the number of shoots with inflorescences in relation to the total number of shoots in each plant fragment.

To assess the genetic make-up of the 24 experimental plant fragments, genomic DNA was manually extracted from epiphyte-free and dried 2-cm-long leaf portions using a standard protocol (nucleo-spin plant II kit, Macherey-Nagel). Individual multilocus genotypes were assessed with a total of 18 microsatellite markers (SSR) taken from the microsatellite set available for the species (Supplementary Table 1). Microsatellites were combined in two different multiplexes and all PCRs were run following Jahnke et al. (2015). Clonal diversity was estimated using the software GenClone (Arnaud-Haond and Belkhir, 2007), while diversity indices (i.e. heterozygosity and allelic richness) and the percentage of polymorphic loci were calculated for each plant fragment (n = 24) and experimental population (n = 2) using the software GenAlex v6.5 (Peakall and Smouse, 2012).

3. Results and discussion

Of the 24 experimental plant fragments used in the study, all but three represented distinct genotypes (Table 1). Two fragments of the same genotype were present in one control tank, while the third was in a heated tank. Both the control and the heated experimental populations showed the same percentage of polymorphic loci (88.89%) and similar genetic characteristics as reflected by the number of different alleles (Na: 2.56 ± 0.26 and 2.72 ± 0.28 for control and heated populations, respectively), the observed heterozygosity (Ho: 0.684 ± 0.097 and 0.688 ± 0.092) and unbiased expected heterozygosity (uHe: 0.455 ± 0.060 and 0.480 ± 0.056). These results indicated that there were no genetic differences between the two sets of samples, also reflected by an unbiased Nei genetic distance between control and experimental plants (uNei = 0.00).

Heated plants showed a significant decrease in leaf growth rate at the end of the exposure period, showing a 24.8% reduction in growth ($F = 10.04$, $p = 0.033$; Fig. 2). The apparent reduction in growth during the recovery period was not significant ($F = 1.56$; $p = 0.280$; Fig. 2).

Eight of the 12 heated plant fragments had inflorescences (Fig. 3), while control plants fragments did not produced any flower (Table 1).

These results provide strong evidence that heat exposure can serve as a primary trigger of flowering in *P. oceanica*. Since all plant fragments were collected haphazardly within the same population and showed no differences in their genetic make-up, it confirms that increased temperatures alone likely induced this response. Indeed, the same genotypes were found in both experimental populations but only bloomed in the heat treatment.

Among heated plants, the intensity of flowering ranged from 8 to 47% and was significantly correlated with allelic richness (n = 7,

Table 1
Characteristics of the distinct plant fragments transplanted in the mesocosm system. For each fragment it is shown: the number of shoots, the percent of flowering shoots, the allelic richness and the heterozygosity. Asterisks indicate the three fragments sharing the same genotype.

Treatment	Tank	Plant fragment	Number of shoots	Flowering shoots (%)	Allelic richness	Heterozygosity
Control	Control A	P1	49	–	1.667	0.667
		P2	45	–	1.611	0.611
		P3	42	–	1.667	0.667
		P4	48	–	1.765	0.765
	Control B	P5	51	–	1.722	0.722
		P6	39	–	1.722	0.722
		P7	44	–	1.722	0.722
		P8	34	–	1.722	0.722
	Control C	P9	45	–	1.667	0.667
		P10	38	–	1.611	0.611
		P11*	56	–	1.667	0.667
		P12*	51	–	1.667	0.667
Heat stress	Heated A	P13*	43	10	1.667	0.667
		P14	45	9	1.647	0.647
		P15	33	–	1.722	0.722
		P16	48	9	1.556	0.556
	Heated B	P17	48	16	1.778	0.778
		P18	49	–	1.667	0.667
		P19	42	27	1.778	0.778
		P20	50	8	1.600	0.600
	Heated C	P21	46	–	1.778	0.778
		P22	39	–	1.667	0.667
		P23	53	12	1.714	0.714
		P24	48	47	1.647	0.647

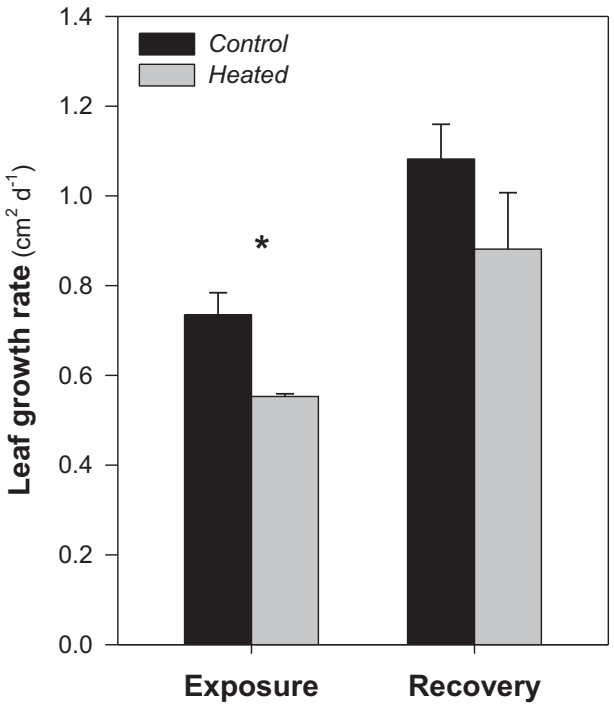

Fig. 2. Leaf growth rates ($cm^2\ shoot^{-1}\ day^{-1}$) of control and heated *Posidonia oceanica* plants after six weeks of heat exposure and six weeks of recovery.

Fig. 3. *Posidonia oceanica* shoots bearing flowers at the end of the experiment.

$r = 0.779$, $p = 0.039$) and heterozygosity (n = 7, $r = 0.783$, $p = 0.037$) but only after removing the genotype showing the strikingly higher flowering rate (47% of flowering shoots, represented with a star in Fig. 4).

This is the first direct experimental evidence that heat stress can effectively trigger flowering in *P. oceanica*. Our results not only demonstrate that thermal stress caused by anomalous temperature as high as those experienced during summer heat waves can induce flowering in *P. oceanica*, but also suggests that genetic diversity has a positive role in enhancing flowering, in accordance with a recent study where genetic diversity of *P. oceanica* patches was positively correlated with flowering intensity (Jahnke et al., 2015).

In a strongly clonal species such as *P. oceanica*, sexual reproduction and seed dispersal can ensure transmission and fixation of epigenetic modifications accumulated during the clonal life of genotypes in response to environmental stress. These mechanisms are expected to play a critical role in the resilience and adaptation of clonal plants to projected conditions of climate change (Dodd and Douhovnikoff, 2016). However, while our results confirm that temperature is an environmental trigger for flowering, whether this is a more general plant response to stressful conditions is difficult to assess. Clearly, *P. oceanica* experiences considerable stress at higher temperatures, as shown by the significant growth inhibition, which is recognized as a clear stress response in higher plants (Lichtenthaler, 1996). Heat induction of flowering could therefore be considered a useful adaptive strategy for the species. By producing flowers, heat-stressed *P. oceanica* plants may be able to invest in producing offspring, thus generating more genetic diversity, which is known to increase plant resilience to disturbances

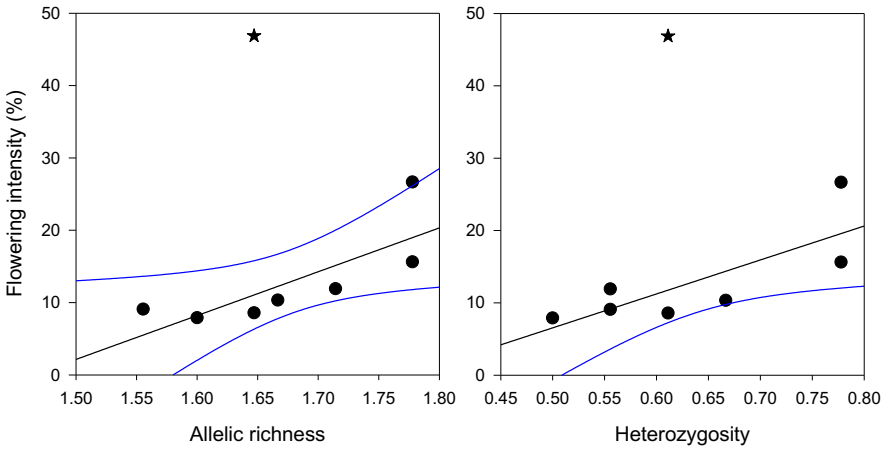

Fig. 4. Results of correlation analysis between flowering intensity and genotype-specific genetic descriptors. Allelic richness (left panel) and heterozygosity (right panel) are shown. Stars indicate the genotype excluded from the correlation analysis.

(e.g. Reusch et al., 2005). Furthermore, for a sessile plant, seed production is the best escape strategy to overcome adverse conditions through seed-driven dispersal (e.g. Blanvillain et al., 2011; Riboni et al., 2014), potentially responsible for the high connectivity between distant *P. oceanica* meadows (Serra et al., 2010). However, the mere production of flowers does not guarantee demographic success since plants still have to overcome several other critical bottlenecks including pollination, fruit and seed set, dispersal and settlement before it translates to viable seedlings. In addition, increased temperatures could influence each of these processes in complex ways, not considered in this study. The relevance of our observations is that *P. oceanica* is capable of this plastic life-history response under heat stress; it remains to be seen if this plasticity confers the species with adaptive demographic advantage in a heating world.

Not all the heated genotypes flowered in our experiment and the number of inflorescences per cultivated plant fragment varied a lot. Factors other than environmental temperature, including genetic, physiological and age related factors, may likely be involved in determining which shoots produce flowers. Since we used apical fragments with a similar size and number of shoots, we can assume that the shoots were of a similar age, thus discarding age-related factors (Balestri and Vallerini, 2003). In contrast, genotypes with higher heterozygosity and allelic diversity flowered more than the rest, although with exceptions. The existence of a correlation between flower density and heterozygosity was previously suggested by Jahnke et al. (2015) in *P. oceanica* patches. In natural conditions, the reason for this could either be that heterozygosity enhanced flowering or, that historical local flowering enhanced heterozygosity. Our results support the first of these two possibilities, although this should be interpreted with caution since our positive correlations hold only after removing the genotype where flowering was much stronger than the others. The existence of exceptions reflects that the genetic factors considered here cannot fully explain the flowering pattern observed. Attributes of clonal plants, such as resource sharing, risk sharing, epigenetic memory, ramet plasticity or genotype-specific response threshold may also play a role in determining shoot-level responses (Dodd and Douhovnikoff, 2016; Latzel et al., 2016; Reynolds et al., 2016).

Heat stress related to global warming has been implicated as triggering a switch between vegetative growth and flowering in many terrestrial plant species (e.g. Balasubramanian et al., 2006). Correlational evidence suggests that warming events could be causing the increase of flowering intensity and frequency in *P. oceanica* meadows, a millenary species forming a keystone marine habitat that dominates infralittoral bottoms of the Mediterranean Sea (Diaz-Almela et al., 2006). *P. oceanica* is characterized by having among the lowest rates of both vegetative and sexual propagation, which makes it one of the most vulnerable to the effects of natural and anthropogenic disturbances. Our results were obtained in controlled mesocosms which has a limited capacity to simulate real natural conditions, however it is worth noting that warming-induced changes in flowering patterns of *P. oceanica* meadows could have relevant ecological consequences for coastal marine ecosystems of the Mediterranean Sea, a region of the world where climatic models have predicted particularly intense global warming effects (Meehl and Tebaldi, 2004).

4. Conclusions

Given that increasing temperature has been implicated in an increase in shoot mortality in this species, the life-history plasticity that *P. oceanica* displays in its reproductive strategies may be a critical mechanism to cope with stressful conditions. First, periodic flowering could significantly increase the diversity of genetic and phenotypic responses to changing environmental conditions. Second, it could serve as a critical mechanism for the population to escape stressful conditions and potentially populate new areas. This plasticity is likely the result of the long ecological history of *P. oceanica* in the Mediterranean, evolving in response to dramatic changes in environmental conditions of temperature and salinity over the last 50 million years and conferring the species with a suite of genetic and phenotypic coping mechanisms to deal with change. Whether this life-history plasticity is sufficient to counter the intensity and pace of global climate change and local anthropogenic disturbance is still uncertain. However, our results show that there is considerable room for optimism as it appears that this species is capable of responding to increasing temperature in more plastic, more complex and potentially more resilient ways than previously imagined.

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Authors contribution

Experimental design: JMR, LM-G, MP, JR, and GP; plant sampling: JB-E, AR-S, YO, NS, JMR, MP, JR, and LM-G; conduction of mesocosm experiment and plant analysis: JB-E, AR-S, RG-M, and LM-G; and all the authors participated to paper writing. All authors have approved the final article.

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